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Genotoxicity Testing in Sediments: Progress in Developing a Transgenic Polychaete Model

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PURPOSE: This technical note describes progress made during calendar year 1999 in the development at the U.S. Army Engineer Research and Development Center (ERDC) of a transgenic marine polychaete worm for testing genotoxicity of sediments. In keeping with the intent of previous communications, this technical note contains protocols for laboratory procedures to aid other researchers who may be similarly engaged in the development of transgenic invertebrate models for research or testing purposes. Protocols are provided for cryopreservation of polychaete sperm and for transgene insertion using electroporation combined with osmotic shock.

BACKGROUND: The reasons for the development of this model are discussed in detail in Technical Note EEDP-01-43 (Inouye, McFarland, and Perkins 1999). Briefly, this work was undertaken in order to address the lack of an appropriate genotoxicity screening assay for dredged sediments. Many of the contaminants (metals as well as organics) commonly encountered in dredged sediments are genotoxic, i.e., capable of damaging genetic material. Open-water disposal of genotoxicants is expressly prohibited in public law. Specifically, Section 103 of the Marine Protection, Research, and Sanctuaries Act of 1972 (MPRSA) at § 227.6 (a) (5) prohibits "known carcinogens, mutagens, or teratogens or materials suspected to be carcinogens, mutagens, or teratogens by responsible scientific opinion," "as other than trace contaminants" if there is a reason to believe such chemicals are present. While the language is less specific in sections of the Clean Water Act regulating dredged material disposal in inland waters (Section 404, CWA 1972, as amended), the intent is similar. Implementation manuals have been written for testing dredged material before disposal under the regulations promulgated in both Sections 103 and 404. However, the recommended tests that are ordinarily performed (bioaccumulation and acute toxicity tests) are incapable of detecting genotoxicity.

Although there are many in vitro assays for genotoxicity available in general toxicology, very few in vivo assays exist that can be applied to sediment-dwelling organisms. The available in vitro assays have numerous limitations, not the least of which is the necessity of testing sediment extracts. The actual fractions of genotoxic chemicals in sediments that are bioavailable to organisms are not represented by extracts, cannot be predicted, and can only be determined in bioassays involving exposure of appropriate organisms directly to the sediments. While in vivo assays are able to discriminate the bioavailable fraction of genotoxicants, those that are available also have severe limitations. Either the type of DNA damage they identify provides insufficient information for determining real probability of eventual development of cancer or of developmental abnormalities, or they must rely on field-exposed animals, or the animal is not intimately associated with sediments in its normal habitat. These limitations led to the research described in this series of technical notes in which transgenic technology is being used to develop a mutation model for sediment genotoxicity testing.

INTRODUCTION: Polychaetous annelids were chosen as the basis for developing a sediment genotoxicity model for several reasons. They are common in most ecological niches of intertidal and subtidal estuarine and marine waters. Many live in direct contact with, and ingest, benthic sediments. Several species, e.g., *Nereis virens* and *Neanthes arenaceodentata*, are widely used in testing sediment toxicity or bioaccumulation of contaminants from sediments. The xenobiotic metabolizing capabilities of some polychaetes are sufficiently well-developed that they are capable of bioactivating PAH compounds, making them suitable surrogates for fish and higher organisms for which the genotoxic potential of sediment-adsorbed PAHs is a major concern. Two species of sediment-dwelling marine polychaetes, *Armandia brevis* and *Neanthes succinea* (Figures 1 and 2),

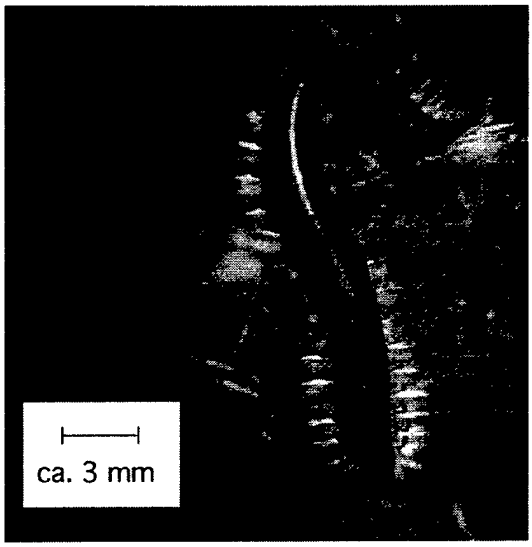

Figure 1. *Armandia brevis*

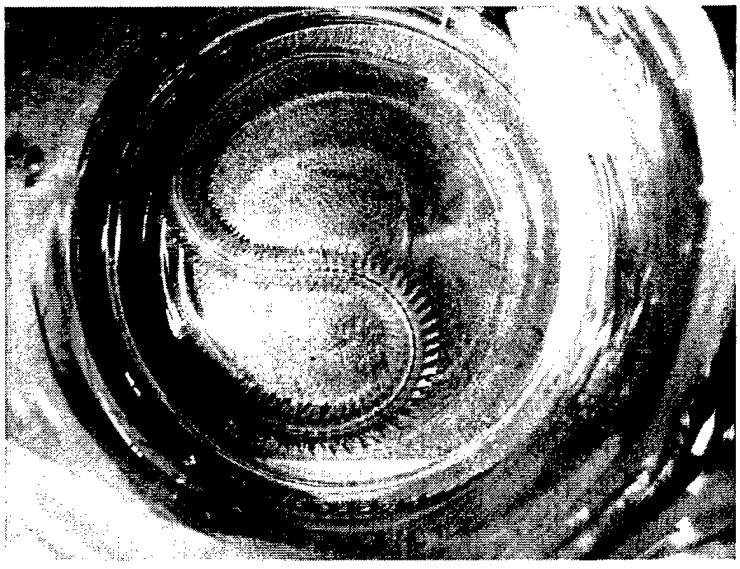

Figure 2. *Neanthes succinea*

were selected for development of a transgenic genotoxicity assay. *Armandia* were collected from Puget Sound, WA, and are limited in their temperature tolerance to about 8-15 °C and to a salinity range of 20-40 °/oo. However, *N. succinea* that were collected from the Salton Sea, CA, can tolerate temperatures ranging from 8 to 30 °C and salinities between 10 and 70 °/oo. Both were first tested for their ability to form DNA adducts after exposure to radiolabeled benzo[a]pyrene. The radioactivity found in the DNA of *N. succinea* was higher than that in *Armandia*, indicating a higher capacity for bioactivation of PAHs in *N. succinea*. Although *Neanthes* shows advantages in temperature range, salinity range, and xenobiotic metabolism over *Armandia,* more information is available on culturing the latter in the laboratory. The reproductive strategy of the worms was also a factor in their selection. For example, unlike *N. arenaceodentata* that reproduces and broods the young in burrows, both *A. brevis* and *N. succinea* reproduce by swarming in the water column, enabling eggs and sperm to be collected relatively easily.

Details of the experimental design are given in Inouye, McFarland, and Perkins (1999). Briefly, a reporter transgene for mutation is inserted into the gametes of the worms prior to fertilization. The larvae produced then contain copies of the reporter transgene in all cells. The transgene is heritable and the worms are kept in continuous culture to provide organisms for genotoxicity testing. Worms are exposed to test sediments for as little as 3-5 days, after which the reporter transgene is recovered from the tissues. The reporter transgene is then amplified in a bacterial culture system to allow detection of mutations and quantification of the mutation frequency. The type of mutations that occur can also be identified because the mutant transgenes are small enough to be easily sequenced.

PROGRESS: Development of culturing techniques for the worms is critical if stable transgenic lines are to be maintained. Currently, both worms are in culture at ERDC. Although methods for *N. succinea* larval care are still being developed, successful laboratory culturing methods are in place for *A. brevis*, with second-generation *Armandia* approaching breeding conditions as of January 2000. One of the major difficulties with culturing these species is that eggs must be fertilized within 24 hr or fertilization will not be successful, and in laboratory cultures, the females and males rarely spawn on the same day. The development of a cryopreservation technique for polychaete sperm was a breakthrough in culturing methods (Appendix A). This method provides long-term storage of sperm from mature males so that eggs may be fertilized when the females release them. Ideally, egg cryopreservation should also be developed, but the methods are much more difficult due to the larger size of the eggs, which makes them more sensitive to damage caused by ice crystal formation during the freezing process. Although several techniques have been tested, none have resulted in viable eggs.

The method developed for transgene insertion (Appendix B) is based on electroporation combined with osmotic shock. Electroporation subjects the sperm to an electrical field in the presence of the transgene, which induces pores in the sperm membrane to open and allows the transgene to enter the cell, while the osmotic shock helps force the transgene through the pores. The sperm are then used to fertilize eggs, incorporating the transgene into the genome of the worm. Early published electroporation methods of transgene insertion often resulted in low production of transgenic organisms. Standard exponential decay electrical pulses typically have high cell survival rates but lower transgene insertion rates than square-wave pulses, while square-wave pulses are very successful at transgene insertion but tend to result in a high percentage of nonviable cells. The

method described in this technical note utilizes electroporation equipment that modulates the electrical field via radio waves (BioRad FlexWave technology), resulting in a modulated square-wave pulse rather than standard exponential decay or square-wave pulses. The resulting electrical pulse retains the transgene efficiency of the square-wave pulses and the high viability of the exponential decay pulse.

Inserting the transgene into *Armandia* was also successful. In order to allow selection of the successfully transformed larvae from non-transgenic larvae, a marker gene was inserted along with the reporter gene. The marker gene provides resistance to hygromycin, a toxin that kills by disrupting protein synthesis. In the presence of hygromycin, larvae without the resistance gene rapidly die, while larvae with the resistance gene are able to survive. Table 1 shows that the survival of untreated control larvae decreases as the concentration of hygromycin to which they are exposed increases, until at 100 µg/ml all control larvae die. At the same concentration, 80 percent of larvae that developed from electroporated gametes survived, indicating successful incorporation of the hygromycin resistance gene.

Table 1
Hygromycin Selection of Transgenic Larvae

Hygromycin µg/ml	Larval Density (% Initial Density)	
	Control	Transgenic
0	86	100
10	79	NT[1]
50	29	50
100	0	80
500	0	NT
1000	0	NT

[1]NT: Not tested.

Currently, the first batch of first-generation transgenic *A. brevis* have passed through the 8-week larval stage, metamorphosed into small worms, and settled into sediment. These worms will reach maturity in 2 to 3 months. A second batch of first-generation transgenic *Armandia* were prepared and will reach the metamorphosis and settlement stage in 4 weeks. The two batches will be interbred to provide a stably transfected culture.

FUTURE DIRECTIONS: Methods for conducting the genotoxicity assay will be developed and optimized, and the dose-related response to several model carcinogens and/or mutagens will be determined. Additional effort will be directed at relating the genotoxicity results to other endpoints. The ability to apply data to ecological risk assessment is a high priority in the Corps of Engineers, and one of the benefits of this model is that a wide variety of data can be gathered with the same species, and potentially even within the same exposure. Recent publications have shown a correlation between genetic markers of exposure and growth, reproduction and survival data in

Daphnia (Atienzar et al. 1999). A series of exposures with *Armandia* and *Neanthes* are in progress that will look at dose-related responses in survival and growth for several model carcinogens and mutagens in order to relate genotoxicity assay results to additional ecologically relevant endpoints.

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Inouye, L. S., McFarland, V. A., and Perkins, E. (1999). "Development of a transgenic model to assess bioavailable genotoxicity in sediments," *Dredging Research Technical Notes Collection* (EEDP-01-43), U.S. Army Engineer Research and Development Center, Vicksburg, MS. *www.wes.army.mil/el/dots/eedptn.html*

APPENDIX A
POLYCHAETE SPERM CRYOPRESERVATION METHODS

OVERVIEW: Cryopreservation requires the use of agents that reduce the formation of ice crystals, the primary cause of cell injury during the freezing process. After testing many common agents, including methanol, glycerol, and DMSO, with concentrations ranging from 5 to 30 percent, a final concentration of 10 percent DMSO worked best. Other factors affecting the success of cryopreservation include the rate of freezing, the storage temperature, and thawing procedures. In the case of polychaete sperm, freezing and storing the samples in the vapor phase of liquid nitrogen preserved viable sperm. Other common methods, including slow constant temperature decrease (-1 °C/min to –80 °C) or flash freezing in the liquid phase of liquid nitrogen, did not produce viable sperm. Rapid thawing and dilution of the thawed sperm in saltwater were also found to be critical to sperm viability.

I. Materials and Reagents

A. Equipment and supplies

Liquid nitrogen dewar
Ice tray/bucket and ice
Various pipettes (10-100 µl range)
Liquid nitrogen
Hanks balanced saline solution, 10X, no phenol red or calcium chloride (Gibco BRL #14185-052)
Dimethylsulfoxide (DMSO), molecular biology grade (Fisher #BP 231-1)
Polypropylene straws (cocktail straws, 2 mm or less diameter opening)
Hemostat
96-well microtiter plate
Disposable glass pasteur pipettes
Cheesecloth

B. Preparation of reagents and supplies

1. 30 g/L HBSS solution:
   Hanks balanced saline solution (10X), without calcium chloride and phenol red. Dilute to 30-32 g/L by adding 2 volumes ultrapure water to 1 volume concentrated HMSS. Store in refrigerator.

2. 20-percent DMSO solution:
   Dilute DMSO by adding 1 volume pure DMSO to 4 volumes 30 g/L HBSS. Store in refrigerator. **CAUTION: SEE MATERIALS SAFETY DATA SHEET- DMSO IS TOXIC AND SHOULD BE HANDLED WITH CARE.**

3. Straws
   Cut the straws into 3.5- to 4-cm lengths, and heat-seal one end. To heat-seal, set up a Bunsen burner or other flame source. Crimp one end of a straw with a hemostat, leaving

1 mm of the straw sticking out. Carefully insert ONLY the 1-mm edge of the straw into the flame, just long enough for the straw to melt. While the straw is still soft, crimp the melted edge either by using another hemostat or by tapping the melted edge on a desktop. Wait for the straw to cool completely before removing the hemostat, or it may fail to seal. Be careful not to insert the straw into the flame too far, or the whole straw can melt. **CAUTION!!! STRAWS WILL CATCH ON FIRE, ESPECIALLY IF LEFT IN THE FLAME TOO LONG!!!**

II. Cryopreservation Methods

A. Obtaining sperm

1. Remove a mature male Armandia from the sediment, being sure to remove all sediment debris.

2. Place the worm in a petri dish, remove all water, and add ~0.25 to 0.50 ml HBSS (30 g/L). The slight osmotic shock (culture water at 32-34 g/L) is usually enough to induce release of the sperm. The calcium-free HBSS media is used to prevent activation of the sperm and to increase the viability of the frozen sperm.

3. If sperm is not released, gently prod the worm with a needle or dissecting probe.

4. If sperm is not obtained after 30 min, the worm is most likely not ready to spawn. Put the worm back in the sediment, and try another male.

5. When sperm is released, put the HBSS-sperm mixture into a microfuge tube and place the tube on ice. Discard the worm, as it dies after spawning.

B. Cryopreservation

1. Be sure everything is set up and ready to go, as minimizing time is critical during this process. The following items should all be within reach: ice tray, containing the vial of sperm; a 96-well plate, and the 20-percent DMSO solution; pipettes, both disposable glass pasteur pipettes and 10-100 µl range pipettes; straws with one end sealed; a flame source to seal the straws; a dewar with liquid nitrogen.

2. Drape the cheesecloth into the dewar containing the liquid nitrogen so that it can suspend straws in the vapor phase of the nitrogen. It is important that the straws do not touch the liquid phase.

3. Gently re-suspend the sperm and pipette 40 µl into several wells of the 96-well plate (one well equals one frozen aliquot).

4. To one well, add 40 µl of the 20-percent DMSO in HBSS solution, and mix. Transfer the mix into a straw. The sample is drawn up into a disposable glass pasteur pipette such that no bubbles are trapped between the tip and the sample. Insert the tip into the straw

until it hits the sealed end. SLOWLY dispense the sample as the pipette is drawn out of the straw. The objective is to get the sample into the straw with no trapped bubbles.

5. Clamp the open end of the straw AS CLOSE TO THE SAMPLE AS POSSIBLE with the hemostat, and flame seal the end. Gently squeeze the tube to confirm a good seal, then drop it into the cheesecloth suspended in the dewar. **CAUTION: GOOD SEALS ARE EXTREMELY IMPORTANT!!! TRAPPED BUBBLES AND BAD SEALS CAN RESULT IN STRAWS EXPLODING UPON THAWING!!!**

6. The process should not take more than 1 min from the addition of DMSO to the freezing of the sample; DMSO is toxic to cells, and longer times between addition and freezing result in reduced viability.

7. Continue the process until all samples are processed.

8. Allow the last sample to freeze for at least 15 min, then transfer the tubes into long-term storage (liquid nitrogen dewar, suspended in vapor phase). Care should be taken to minimize the transfer time to prevent sample thawing.

C. Thawing and use of frozen sperm.

1. Remove the desired number of straws from the liquid nitrogen dewar, and IMMEDIATELY place the straws in a beaker of water (room temperature). **CAUTION: BAD SEALS AND TRAPPED BUBBLES MAY RESULT IN STRAWS EXPLODING UPON THAWING. USE APPROPRIATE SAFETY GEAR!!!**

2. After the straws have thawed, which takes only a few seconds, the sample should be removed from the straw and diluted with saltwater as quickly as possible. Cut off one end of the straw, and place over a beaker of saltwater with the open end down. Cut off the other end, and rinse the contents into the beaker. The time taken for thawing and dilution should be kept at the absolute minimum to ensure viable sperm.

3. Diluted sperm can now be used to fertilize eggs.

APPENDIX B
TRANSGENE INSERTION METHODS

OVERVIEW: This method combines two different techniques, electroporation and osmotic shock. Sperm cells are first subjected to a hypersaline buffer, which results in dehydration of the cell. The transgene, in a hyposaline solution, is then added to the dehydrated sperm and subjected to an electrical pulse. The combination of the opening of pores in the cell membrane due to the electrical pulse and the sudden decrease in salinity of the media causes the media (and transgene) to enter the cells.

I. Materials and Reagents

A. Equipment and supplies

BioRad GenePulser II system
BioRad GenePulser II RF Module
0.1-mm gap cuvettes for electroporator
Various pipettes (1- to 10-µl range, 10- to 100-µl range, 10- to 250-µl range)
Hanks balanced saline solution, 10X, no phenol red or calcium chloride (Gibco BRL #14185-052)
Transgene,[1] approximately 100 µg/ml in ultrapure water
Hygromycin (Sigma #7772)

B. Reagent and supplies preparation

1. HBSS solution (30 g/L):
   Hanks balanced saline solution (10X), without calcium chloride or phenol red. Dilute to 30-32 g/L by adding 2 volumes ultrapure water to 1 volume concentrated HMSS. Store in refrigerator.

C. Obtaining *Armandia* gametes

1. Sieve worms from sediment. Separate the females (golden) from the males (ivory). Return males to sediment for later use after a female spawns.

2. Place females in ~10-20 ml clean saltwater (32-34 g/L) with an airstone or bubbler to provide aeration. Females must be allowed to spontaneously spawn or the eggs are nonviable. Discard the worm after it spawns. For electroporation, the eggs from one female are divided up into 10 equal batches, each of which is brought up into 50 ml of saltwater (32-34 g/L).

3. After a female spawns (this can take up to 2 weeks), remove a mature male polychaete from the sediment, being sure to remove all sediment debris. Do not use cryopreserved

[1] Transgene construction: see, e.g., Technical Note EEDP-01-43 (Inouye, McFarland, and Perkins 1999) *www.wes.army.mil/el/dots/eedptn.html*

sperm when inserting transgenes, since the electroporation is best conducted on sperm that have not yet been activated in saltwater.

4. Place the worm in a petri dish, remove all water, and add ~0.25 to 0.50 ml HBSS (30 g/L). The slight osmotic shock (culture water at 32-34 g/L) is usually enough to induce release of the sperm. Calcium-free media are used to prevent the activation of the sperm prior to electroporation.

5. If sperm is not released, gently prod the worm with a needle or dissecting probe.

6. If sperm is not obtained after 30 min, the worm is most likely not ready to spawn. Put the worm back in sediment, and try another male.

7. When sperm is released, put the HBSS-sperm mixture into a microfuge tube and place the tube on ice. Discard the worm, as it dies after spawning.

II. Transgene Insertion

A. Setting up the electroporation system (the following directions apply to the BioRad GenePulser II with the BioRad GenePulser RF module)

1. Follow the manufacturer's directions for turning the equipment on.

2. Set the GenePulser RF Module parameters as follows:
   20 volts
   100-percent modulation
   5-ms duration
   5 bursts
   1-s interval

B. Electroporation

1. Pipette 38 µl of diluted HBSS (30 g/L) into the cuvette (0.1-mm gap).

2. Pipette 2 µl of sperm in HBSS into the cuvette and mix.

3. Add 10 µl of concentrated stock HBSS (10X), mix, and allow the sample to sit for 5-10 sec (dehydrating process). Salt concentrations of this solution are approximately 42 g/L; higher concentrations result in loss of *Armandia* sperm viability.

4. Add 20 µl of the transgene (in ultrapure water), mix and IMMEDIATELY electroporate (rehydration and electroporation). Salt concentrations of this mixture are 30 g/L; lower concentrations result in severe loss of *Armandia* sperm viability.

5. Transfer the sperm into a prepared egg suspension (see Section 1.C.2 for preparation of egg suspension), rinsing the cuvette with 40 µl of saltwater (culture water, 32-34 g/L) to ensure complete transfer.

6. Combine the fertilized eggs into a 2-L beaker with a gently bubbling airstone, and place in an 11-13 °C waterbath. If hygromycin is used to selectively kill off non-transgenic larvae, begin treatment at week 6. Add hygromycin to a final concentration of 1 µg/ml, and treat until ready for larval settlement (8 weeks).